

5-2010

GROWTH AND HYDROGEN PRODUCTION FROM CARBOHYDRATES AND SWITCHGRASS BY THERMOTOGA NEAPOLITANA

Divya dharshini Chandrasekaran
Clemson University, divya.dharsh@gmail.com

Follow this and additional works at: https://tigerprints.clemson.edu/all_theses

 Part of the [Microbiology Commons](#)

Recommended Citation

Chandrasekaran, Divya dharshini, "GROWTH AND HYDROGEN PRODUCTION FROM CARBOHYDRATES AND SWITCHGRASS BY THERMOTOGA NEAPOLITANA" (2010). *All Theses*. 847.
https://tigerprints.clemson.edu/all_theses/847

This Thesis is brought to you for free and open access by the Theses at TigerPrints. It has been accepted for inclusion in All Theses by an authorized administrator of TigerPrints. For more information, please contact kokeefe@clemson.edu.

GROWTH AND HYDROGEN PRODUCTION FROM CARBOHYDRATES AND
SWITCHGRASS BY *THERMOTOGA NEAPOLITANA*

A Thesis
Presented to
The Graduate School of
Clemson University

In Partial Fulfillment
Of the Requirements for the Degree
Master of Science
Microbiology

by
Divya Dharshini Chandrasekaran
May 2010

Accepted by:
Dr. J. Michael Henson, Committee Chair
Dr. Melissa. B. Riley
Dr. T.R. Jeremy Tzeng

ABSTRACT

Thermotoga neapolitana is an anaerobic thermophilic marine bacterium that has been reported to degrade cellulose. This hyperthermophilic bacterium grows at 77°C and could be used in large scale applications because of its ability to withstand extreme conditions. The aim of this study was to analyze the growth and production of hydrogen by *T. neapolitana* when grown on various carbon sources including the bioenergy crop switchgrass. Switchgrass was provided by the Clemson University Pee Dee Research and Education Center and was milled to about 4mm in size. Switchgrass was then added to an anaerobic medium and inoculated with *T. neapolitana*. Headspace gas analysis indicated production of about 5% hydrogen from switchgrass. The physical nature of the switchgrass was visibly altered and dry weight analysis indicated that about 9.4% of switchgrass was degraded. Treatment to remove lignin did not improve conversion efficiency. Heat treatment of the switchgrass by autoclaving did not have an effect on the hydrogen production. Increasing concentrations of switchgrass in the medium led to a corresponding increase in turbidity and the headspace hydrogen percentage. Potential effects of light on the growth and hydrogen production of *T. neapolitana* were also evaluated. When grown on carbohydrates in the dark, the growth as measured by turbidity was greater than when grown in room light conditions.

ACKNOWLEDGEMENT

When I joined Clemson in fall 2007, I started at the Food Science Department. Early that semester when I met Dr Mike Henson, he had not started a lab yet. But he agreed to accept me as his first graduate student the following spring. He helped me get funded in my first semester and the subsequent semesters. I then switched to Microbiology in spring 2008 and from then on, I have had the honor of working with Dr Henson. Right from day one when we started out sharing space with Dr Drapcho's lab, and then finding space for our own lab, buying equipments, I m proud to say that I am the only one here to have seen an advisor wash glassware with his graduate student. Ever since, he has been my mentor – sometimes behind me pushing me to do the right things, most of the times ahead of me showing the path, and always beside me supporting me even when I came up the most ridiculous of ideas. Thank you, Dr Henson for your wisdom, guidance, and your patience. Above all, thank you for believing in me when I was just a fresh-off- the-boat graduate student and for being the coolest mentor anyone could get.

Thank you Dr Jeremy Tzeng and Dr Melissa Riley for being on my committee and for all the enthusiasm and interest you showed in my research. Dr Tzeng, thank you for your expertise on bacterial techniques whenever I had trouble with *Thermotoga*. Thank you Dr. Riley for the way you almost magically willed our chromatography to work and for helping me with my data.

I would like to thank Dr Caye Drapcho and Dr Terry Walker for letting me borrow their equipments. Thank you, Abhiney and Xiaohui Yu for teaching me basics of growing *Thermotoga*. I would also like to thank Kirk Fowler for being a very persistent technician in our lab and for assisting me with my work

for almost a year. Thank you Renuka, Chris, Sandra, Abhiney, Kristin and Arpan for all the scientific inputs and the not-so-scientific pep talks and good times. I would also like to thank the undergraduate students who have worked with me – Stephanie, Meg, Katie and Audrey, without whom I would ve spent longer nights and days in the lab.

I would also like to thank Darryl Krueger for his help with electron microscopy. A special note of thanks to the administrative staff of Biological Sciences for handling all my paper work over these years.

Finally I would like to thank U.S Department of Energy and Sun Grant for funding this project and Pee Dee Research Centre for their valuable resources.

DEDICATION

This thesis is my first active contribution to the world of Microbiological Research and I would like to dedicate this to my parents, friends and family.

Thanks Daddy and Ma, for taking me seriously when I said I wanted to do Food Science and then changed my mind six months later and said – I m switching to Microbiology. Thank you for waiting patiently for me to graduate, and supporting me throughout my life. Thanks to my sister Ramya, her husband Prakash and my new born niece Riya for being here in the U.S and providing me with a home that I can come crying to every now and then. I would also like to thank my classmates from India and my friends here who have been there for me no matter what and for all the good times.

This dedication would be incomplete if I don't thank my bacterium *Thermotoga neapolitana* for having spent the most time with me in these two and a half years, for being the hero of my research and for all the fun we had.

TABLE OF CONTENTS

ABSTRACT	ii
ACKNOWLEDGEMENT	iii
DEDICATION	v
LIST OF FIGURES	viii
LIST OF TABLES	x
INTRODUCTION AND LITERATURE REVIEW	1
Need for Alternative Fuels.....	1
Switchgrass	2
Bio Hydrogen.....	5
<i>Thermotoga neapolitana</i>	6
<i>Thermotoga neapolitana</i> Cellulases	7
MATERIALS AND METHODS	9
Organism and Culture maintenance	9
Cultivation medium.....	9
Pretreatment effects on hydrogen production.....	10
Effect of light and darkness on growth	10
Headspace Analysis methods	11

RESULTS AND DISCUSSION	13
<i>T. neapolitana</i> hydrogen production from switchgrass	13
Effect of pretreatment and autoclaving of switchgrass on hydrogen production.....	14
<i>T. neapolitana</i> – Growth on Filter paper.....	15
Effect of light on <i>T. neapolitana</i> s growth in carbohydrates	17
CONCLUSIONS AND FUTURE STUDIES	19
REFERENCES.....	30

LIST OF FIGURES

Fig.1. Trend in crude oil and petroleum imports from 1949 - 2008 in the United States.	20
Fig.3. Fermentation of carbohydrate rich substrates to fermentation products like hydrogen, carbon dioxide and organic acids and alcohols (Hallenback <i>et al.</i> , 2009).	21
Fig.4. Metabolic pathway of glucose being fermented to 2 acetate, 2 CO ₂ and 4 H ₂ by <i>Thermotoga maritima</i> (Schroder <i>et al.</i> , 1994).	22
Fig.6. Glucose medium – uninoculated control and inoculated test sample.	23
Fig.7. Switchgrass uninoculated control and inoculated test sample.	24
Fig.8. Hydrogen and carbon dioxide production from increasing concentrations of switchgrass.	24
Fig.9. Filtered, dried switchgrass (uninoculated control on the left and inoculated on the right)	25
Fig.10. Carbohydrates released when non pretreated non autoclaved switchgrass was washed	25
Fig.11. Carbohydrates released when non pretreated autoclaved switchgrass was washed.	26
Fig.12. Yellow color formed in <i>T. neapolitana</i> culture grown on filter paper, on the left. On the right is the uninoculated control.	26
Fig.13. Percentage of headspace hydrogen produced from increasing concentrations of filter paper and medium with and without yeast extract and filter paper.	27
Fig.14. Acetate production from filter paper and yeast extract over a period of five days by <i>T. neapolitana</i>	27

Fig.15. Growth rate of *T. neapolitana* when grown in the presence and absence of light

28

LIST OF TABLES

1. Modified table showing different methods of hydrogen production and their corresponding hydrogen yields (Levin *et al.*, 2003) 29
2. Dry weight differences between control and inoculated switchgrass 29

INTRODUCTION AND LITERATURE REVIEW

Need for Alternative Fuels

Decrease in availability of fossil fuels has led to an increase in the cost of conventional fuel sources such as crude oil. The rising awareness of depletion of fossil fuels and concern for environmental well being has stimulated research in alternative fuels throughout the world. Fig.1. shows the trend in crude oil and petroleum product imports from 1949 - 2008 in the United States.

This current unsustainable energy scenario demands efficient and practical solutions to meet the world's energy needs and that is why the United States Department of Energy (DOE) began investigating biological resources for energy production. Lignocellulosic biomass is an important source of biofuels. Its abundance in the form of bioenergy crops, forage waste, and agricultural waste makes it a potential source of alternative energy. Annual biosynthesis of cellulose by plants and marine algae occurs at a rate equivalent to more than four times the world's annual energy consumption (Nowak *et al.*, 1995). Utilization of biomass may also aid in waste disposal problems and other related issues.

Many plant species were considered for use as biomass for energy by DOE when they developed interest in biomass resources in 1977 and co-funded work on woody crops, sugar cane, and tropical grasses along with U.S Department of Agriculture. Oak Ridge National Laboratory (ORNL) was asked by DOE to work on the selection of plants for the woody biomass projects effort. In 1984, DOE funded ORNL to develop an Herbaceous Energy Crops Program (Wright, 2007). Six universities and one private company were selected to participate in the herbaceous screening projects. The universities included Cornell University, Virginia Polytechnic Institute and

State University, Auburn University, Purdue University, Iowa State University, and North Dakota State University. The seventh research group was a small company in Ohio, named Geophyta. These universities conducted a survey of crops and plants, annual and perennial – and came up with several suggestions. Switchgrass was the choice energy crop for six out of seven projects. Sorghum was also suggested as one of the best energy crops but other studies indicated that sorghum performs better in the Midwest while switchgrass performed better in the Southeast (Turhollow *et al.*, 1990). Some of the criteria for the selection were yield, sustainability (economic and environmental), adaptability, and minimal soil loss. Wright (2007) describes how the criteria for selection have been different in the 1980s and 1990s. The criteria in the 1980s were profitability on marginal land, adaptability, minimal soil loss, high yield potential and yield reliability. In the 1990s, the criteria were profitability on productive land, feedstock composition, reliable stand establishment, reliable low-cost propagation and soil carbon sequestration.

Switchgrass

At present, the US produces most of its bioethanol from corn. Continued usage of corn will have significant effects on arable land requirements (Sun and Cheng, 2002) and will result in higher corn prices that will negatively impact the food and feed industries. This in turn could result in reduced exports of animal products (Elobeid *et al.*, 2007). These concerns have led to the need for alternative nonfood crops for use as energy crops.

Switchgrass (*Panicum virgatum* L.) is a perennial warm-season grass native to North America. (Fig.2). It can grow up to ten feet tall and was identified by the US DOE as a model herbaceous energy crop. Switchgrass shows promise due to its high productivity, suitability for marginal land quality, low water and nutritional requirements (Keshwani *et al.*, 2009). Switchgrass is made up of

lignocellulose which contains lignin (17%), hemicellulose (27%) and cellulose (36%). (Keshwani *et al.*, 2009). For the breakdown and subsequent fermentation of switchgrass, lignin has to be removed as one of the steps called pretreatment. This step makes the cellulose and hemicellulose accessible to enzymes, reduces carbohydrate losses and prevents formation of by-products that inhibit subsequent hydrolysis and fermentation steps (Keshwani *et al.*, 2009). The lignin removed can be used for different applications (Wyman *et al.*, 1994).

Prior to lignin removal, pretreatment of switchgrass can be accomplished in many ways – physically, chemically or biologically. Physical pretreatment can be done by grinding, milling or chopping. There have been many studies on physical pretreatment of switchgrass and their effects (Schell and Harwood, 1994; Sun and Cheng, 2002; Mani *et al.*, 2004; Yu *et al.*, 2006; Igathinathane *et al.*, 2008). One such study by Bridgeman *et al.* (2007) reported that for particle sizes smaller than approximately 900 μm , cellulose content was 13.4% lower than for larger particles. The losses in lignin and hemicellulose were appreciably less (3.43% and 4.74%, respectively). Thus, physical pretreatment could lead to over reduction of size which causes significant carbohydrate losses which ultimately results in less reducing carbohydrates and reduced biofuel yield.

Chemically, pretreatment can be accomplished in many ways using acids, alkali, ozone, peroxides and organic solvents (Vidal and Molinier, 1988; Tarkow and Feist, 1969; Wood and Saddler, 1988; Chum *et al.*, 1988; Thring *et al.*, 1990; Wu and Lee, 1997; Chang *et al.*, 1997). Dilute acid pretreatment is the most commonly studied pretreatment method. Acid pretreatment primarily acts by solubilizing the hemicellulose and reducing the crystallinity of cellulose. The lignin content is retained for the most part, whereas alkali pretreatments target the removal of lignin. Sometimes hemicelluloses are also removed during alkali pretreatment (especially at high temperatures) because of their random amorphous structure (Keshwani, 2009). Up to 99% delignification has been

reported using a combined ammonia–hydrogen peroxide percolation pretreatment at 170 °C (Kim and Lee 1996). There are other methods that were studied like calcium hydroxide pretreatment (Chang *et al.*, 1997). A combined microwave–alkali pretreatment of switchgrass was suggested by Keshwani *et al.*, 2007.

Biological pretreatment can be performed by using microorganisms like white- rot fungi (Ander and Eriksson, 1977). Because of the slow rate, biological pretreatment is not generally used.

Once a suitable pretreatment method is performed, chemical and/or physical, the switchgrass is then washed and pH- adjusted to make it conducive for hydrolysis. Hydrolysis of cellulose is the process by which cellulose is converted to fermentable carbohydrates. Enzymatic hydrolysis is an environmentally friendly way of achieving this. Generally fungal cellulases are utilized for this purpose although many bacteria like *Clostridium*, *Cellulomonas* and *Bacillus* (Bisaria, 1998), produce cellulases. Cellulases are a family of enzymes that in a coordinated manner convert the non-soluble polymer of cellulose into water soluble carbohydrates. There are several enzymes involved in this conversion process, which are as follows : endo- β -glucanases (1, 4- β -D glucan 4- glucohydrolase [EC 3.2.1.4]), exoglucanases (1,4- β -Dglucan cellobiohydrolase [EC 3.2.1.91]), glucan glucohydrolases (1,4- β -D-glucan glucohydrolase [EC 3.2.1.74]), and β -glucosidases (β -D-glucoside glucohydrolase [EC 3.2.1.21]).The endoglucanases randomly hydrolyze internal glycosidic linkages, resulting in a rapid decrease in polymer length and a gradual increase in the reducing sugar concentration. The exoglucanases also referred to as cellobiohydrolases hydrolyze cellulose chains by removing cellobiose either from the reducing ends or the non reducing ends, which results in rapid release of reducing carbohydrates but little change in polymer length. The cellobiose is then converted to glucose monomers by β -glucosidases (Clarke, 1997). The cellulolytic enzyme system of *T. neapolitana* has been described (Bok *et al.*, 1998).

Bio Hydrogen

Hydrogen has been proven to be a cleaner and greener source of energy to run motor powered vehicles (Hydrogen production - U.S. Department of Energy official website, (accessed April 20, 2010)). The most popular methods of hydrogen production have been from electrolysis and steam (van Ooteghem *et al.*, 2002, Carere *et al.*, 2008). Microbial method of hydrogen production is a relatively new field and can be achieved in many ways like direct biophotolysis, indirect biophotolysis, photo-fermentations, and dark-fermentation (Levin *et al.*, 2004; Das *et al.*, 2001). Photo fermentation is the process in which a non sulfur purple photosynthetic bacterium uses captured solar energy to produce ATP and high energy electrons that reduce ferredoxin. This in return reduces the proton to hydrogen with the help of nitrogenase (Hallenbeck *et al.*, 2009).

Dark fermentation is the process by which anaerobic bacteria produce hydrogen and usually carbon dioxide by fermenting carbohydrate sources. Thermophilic anaerobes are common dark fermenters. Some mesophilic bacteria like species of *Enterobacter*, *Bacillus*, and *Clostridium* have been shown to produce hydrogen. The yield of hydrogen varies according to the metabolic pathway of the bacterium. For instance, in *T. neapolitana*, acetic acid is made along with hydrogen, and in this case the yield of hydrogen has a theoretical maximum of 4 moles/mol glucose (van Ooteghem *et al.*, 2004). The stoichiometry is as follows.



Hydrogen yield has been shown to be higher in organisms that produce acetic acid as opposed to propionic acid and reduced end products like alcohol (Hallenbeck *et al.*, 2004). Thus

different methods of production of hydrogen have been evaluated and dark fermentation has been shown as a promising method of producing bio hydrogen, considering the yield of hydrogen, as shown in Table 1. Table. 1 also shows that an anaerobic, thermophilic acetic acid producing bacterium is the most suitable for hydrogen production.

Thermotoga neapolitana

Thermotoga neapolitana is an extremophilic bacterium first isolated in the bay of Naples, Italy in 1986 (Jannasch *et al.*, 1988). *Thermotoga* species have been found in diverse locations around the globe (Huber *et al.*, 1986; Jannasch *et al.*, 1988; Jeanthon *et al.*, 1995; Ravot *et al.*, 1995; Fardeau *et al.*, 1997; Takahata *et al.*, 2001; Balk *et al.*, 2002). *Thermotoga* species are Gram negative rods, non motile, strictly anaerobic and grow at an optimum temperature of 80°C and optimum pH of 7. They have an outer sheath like structure that balloons over the ends of the cell. The cell length can be between 0.5 to 11µm. This bacterium utilizes the carbohydrates xylose, glucose, sucrose, maltose, lactose, and glycogen as substrates (Jannasch *et al.*, 1988). More than twenty different members in more than five different genera are currently known in this class *Thermotogales* (Kelly, 1994).

Thermotoga have been isolated from a variety of environments; freshwater and marine hot springs, hot sulfur springs, near the mouth of marine black smokers, and hot oil wells. All of these organisms have been isolated from environments where the temperature is significantly elevated. Most of the *Thermotogales* can withstand elevated pressures as well and can grow at atmospheric pressure as long as the temperature is elevated (Jannasch *et al.*, 1988; Kaplan *et al.*, 1988; Kelly *et al.*, 1994). *Thermotoga neapolitana* is a marine bacterium; hence it has tolerance to salinity. The ability to grow in extreme conditions is favorable in an organism that is used industrially.

Thermotoga neapolitana has been primarily utilized for its hydrogen production efficiency. Yu (2008) reported that *T. neapolitana* accumulated 28%-30% hydrogen in the headspace, using glucose as carbon source after 20 hours incubation. *Thermotoga maritima* completely ferments 1mole glucose as a carbon and energy source to 2 moles acetate, 2 moles carbon dioxide and 4 moles hydrogen through Embden-Meyerhof pathway (Schroder *et al.*, 1994; Schonheit and Schafer, 1995).

Thermotoga neapolitana can use simple or complex carbohydrates as carbon source (Huber and Hannig, 2006;; Yu 2008; Nguyen *et al.*, 2008). Vrije *et al.* (2002) reported *Thermotoga elfii* utilizes pretreated *Miscanthus* and produces a significant amount of hydrogen. Yu (2008) reported that *Thermotoga neapolitana* can utilize cellulose as its carbon source.

***Thermotoga neapolitana* Cellulases**

Carbohydrate uptake in *T. neapolitana* is primarily by a binding protein dependent ABC transporter (Nanavati, 2002). When grown on lignocellulosic biomass, *T. neapolitana* needs to break down the insoluble lignocellulose to fermentable carbohydrates that are then taken up into the cell. This process is achieved by breaking down the cellulose content by utilizing a variety of enzymes including cellulases.

Thermotoga are also important sources of other thermostable glycosyl hydrolases like xylanases, xylosidases, amylases, b-glucosidases, mannanases, and galactosidases (Nelson KE *et al.*, 1999; Bronnenmeier *et al.*, 1995; Duffard *et al.*, 1997; Gabelsberger *et al.*, 1993; King *et al.*, 1998). Cel A and Cel B are two endoglucanases isolated and purified from *T. neapolitana* and is shown to have

an enzyme activity of about 1,219 and 1,536 U/mg, respectively, for carboxymethyl cellulose. Both these enzymes are induced by cellobiose and repressed by glucose (Bok *et al.*, 1996).

This study is unique in its use of this particular bacterium and this particular substrate to make hydrogen. Switchgrass is being evaluated by U.S. DOE as a promising feedstock primarily for bioethanol and butanol production. Results of this study throw light on switchgrass as a bioenergy substrate and *T. neapolitana*'s ability to ferment various carbohydrate sources. The aim of this study is to study the degradation of various complex substrates, switchgrass in particular to hydrogen using *Thermotoga neapolitana*, a thermophilic anaerobic marine bacterium that grows at 70-80°C. *T. neapolitana* ferments simple and complex sugars to hydrogen, acetate and carbon dioxide. It has been proven to be a fermentative hydrogen producer that produces up to 25% hydrogen from glucose. Since switchgrass is a lignocellulosic biomass, the breakdown requires pretreatment followed by enzymatic hydrolysis to convert the cellulose into fermentable sugars. CelA and CelB are two thermostable cellulases that have been isolated from *T. neapolitana*. The fact that these enzymes are thermophilic makes it even more lucrative in an industrial process since issues like sterility maintenance and salt tolerance are not a concern.

MATERIALS AND METHODS

Organism and Culture maintenance

Thermotoga neapolitana was obtained from DSMZ (the German Resource Centre For biological Material). *T. neapolitana* was maintained on the medium described by Van Ooteghem *et al.* (2002). The medium contains 1.0g of NH₄Cl, 0.3g of K₂HPO₄, 0.3g of KH₂PO₄, 0.2g of MgCl₂·6H₂O, 0.1g CaCl₂, 10.0g of NaCl, 0.1g of KCl, 1.14g of cysteine HCl. H₂O, 2.0g of yeast extract, 2.0g of Trypticase, 10.0ml of vitamin solution (DSM media 141), 10.0 ml of trace element solution (DSM media 141), and 0.121g of trizma base in 1.0 L of distilled water. The initial pH of the medium was adjusted to 8.0 using 5N NaOH. The organism was preserved at 4°C.

Cultivation medium

The composition of the medium used for experiments was the same as described for culture maintenance. Switchgrass, obtained for the Clemson University Pee Dee Research and Education Center, South Carolina, was cut into 18 to 24 inch portions and was milled to particles of 4 mm in size using a Thomas Wiley mill (Thomas Scientific Model 4). The bacterium was grown in either 150 mL serum bottles or 20 mL serum tubes. For the serum bottles, 50 ml of the medium was prepared and the growth substrate such as switchgrass, delignified switchgrass, or carbohydrates was added at a final concentration of 5 g/L, unless otherwise noted. All treatments were run in triplicates.

Bottles were sparged with nitrogen, at outlet pressure of 5 psi, for atleast 5 minutes. The medium was inoculated with 2 ml of *T. neapolitana* culture using sterile syringes. The culture was

incubated in an orbital shaker (New Brunswick) at 121 rpm and 77°C whenever the substrate was a soluble sugar. When the samples contained switchgrass or filter paper, cultures were not shaken.

Pretreatment effects on hydrogen production

The lignin-removal pretreatment of switchgrass consisted of placing 45 g of switchgrass in a liter bottles followed by addition of 210 mL of 15% ammonium hydroxide. Bottles were incubated in a water bath at 60°C for 12- 16 h. After incubation, the switchgrass was washed with distilled water until the pH reached 7.0. After autoclaving, the switchgrass was then dried in an oven at 70°C. The effect autoclaving on *T. neapolitana* growth was analyzed by comparing non autoclaved medium with autoclaved medium containing switchgrass. The switchgrass was then filtered using a 0.2µm filter and a vacuum system. The filtrate was then inoculated with *T. neapolitana* to study the effect of autoclaving.

For the experiments with filter paper, Whatman No.1 filter paper was used in the form of strips of about 5 cm in length.

Effect of light and darkness on growth

To assess the potential effects that light may have on the growth of *T. neapolitana*, anaerobic medium was prepared as described previously and autoclaved. Sugar solution was filtered through 0.2 µm filters and added prior to inoculation, to the medium. The growth curve experiments were done in serum tubes with 10 mL of medium. The samples to be kept in dark were wrapped in aluminum foil, while the samples in light were kept in the presence of ambient light. Optical Density (OD) was measured every 2 hrs at 600 nm, for 12 hours, using a Thermoscientific spectrophotometer model Spec 20.

Headspace Analysis methods

Serum bottles/tubes were allowed to cool to 25°C, before headspace analysis. This cooling step was done to allow the majority of the water vapor present in headspace to condense, thus reducing its interfering with the analysis.

Total headspace pressure

The pressure of the gas in the headspace of each reactor was measured with Traceable manometer (Fisher Scientific) after the reactor had cooled to room temperature. According to ideal

gas law, the hydrogen concentration was calculated using the following equation,
$$C_{H_2} = \frac{P_{H_2} V_1}{RT} * \frac{1}{V_2} .$$

C_{H_2} is the hydrogen gas concentration (mol H₂/L medium), P_{H_2} is the hydrogen partial pressure (atmospheres), V_1 is the volume of headspace (L), T is the temperature (°K), R is the universal gas constant (0.0821 L·atm/(mol·K)), V_2 is the volume of medium (L).

Hydrogen Analysis

Hydrogen was quantified by collection of headspace with a gas-tight syringe, injection of 0.2 mL into an Agilent 7890A gas chromatograph, inlet at 250°C split 10:1, 8.63 psi, total flow 102 mL/min, HP-PLOT Molesieve column (30 m, 0.32 mm, 3μM) set to a constant pressure of 8.8671 psi, with the oven temperature at 50°C, held for 1.5 minutes then to 150°C at 50°C /min and held for 2 minutes. The detector was a thermal conductivity detector (TCD) at 250°C with a reference flow 20 mL/min and makeup flow at 2 mL/min. Helium was used as the carrier gas.

Carbon dioxide Analysis

Carbon dioxide in the headspace was measured by injecting 0.5ml of the headspace gas into

gas chromatograph (Agilent 7890A) with GS-CarbonPLOT column (J&W) with an oven temp of 35°C, injector at and TCD at 150°C. Helium at 30ml/min was used as the carrier gas.

HPLC

Carbohydrates were analyzed using a Shimadzu high performance liquid chromatography. Pump LC-20AT was set to 0.600 mL flow with deionized water degassed with helium as mobile phase. The CTO-20A oven with a Bio-Rad Aminex HPX-87P column was set operating at 80°C, carbohydrates were detected using RID-10A refractive index detector, and CBM-20A controller. Samples were removed from the growth vessels, centrifuged and filtered through 0.2 µm filters prior to sample injection using a refrigerated autosampler.

Acetate Analysis

A Short FFAP column was used to measure acetate concentration on an Agilent Gas Chromatograph equipped with a Flame Ionization Detector (FID). 1 µL sample is injected into the column. The inlet pressure was 16.185 psi, the total flow was 52.5 mL/min, and the injector was at 230°C. The flow through the column was 0.6447 mL/min. The initial temperature of the column was 60°C and it was held for 1 minute, and then increased to 120°C at a rate of 3°C/min. The temperature was then increased to 160°C at a rate of 65°C/min, increased to 220°C at 15°C/min and held for 2 min. The hydrogen flow rate was 30 mL/min, the air flow rate was 400 mL/min, and the makeup gas (helium) flow rate was 25 mL/min. The sample was acidified prior to analysis by adding 50 µL of 10% formic acid to 450 µL of sample.

RESULTS AND DISCUSSION

***T. neapolitana* hydrogen production from switchgrass**

Experiments were conducted to evaluate whether *T. neapolitana* grows on switchgrass. Since medium containing switchgrass is non homogenous, growth measurements using direct cell count or other conventional methods was not feasible. Growth was measured in terms of amount of headspace hydrogen produced. When *T. neapolitana* was grown in medium containing 5 g/L glucose, 15% headspace hydrogen was produced and when grown on 5g/L switchgrass, 5% headspace hydrogen being produced. (Fig .5)

In order to ensure that the hydrogen was being produced from switchgrass, *T. neapolitana* was grown in medium that was devoid of switchgrass and it was observed that no hydrogen was being produced. *T. neapolitana* was then grown in medium containing increasing concentrations of switchgrass (5, 10 and 25 g/L). Fig.6 and Fig.7 show turbidity in medium when *T. neapolitana* was grown on glucose and switchgrass respectively.

Headspace hydrogen and carbon dioxide production were found to increase with increasing concentrations of switchgrass (Fig.8). To further visually observe the consumption of switchgrass, dry weight analysis of switchgrass was performed by filtering followed by drying in an oven at 60°C degrees over night. Dry weight analysis showed 9.4% degradation of switchgrass (Fig.9, Table 2.). These results indicate that *T. neapolitana* can degrade switchgrass resulting in the generation of hydrogen gas.

Effect of pretreatment and autoclaving of switchgrass on hydrogen production

Switchgrass is composed of 17% lignin. For hydrolysis to occur lignin needs to be removed or reduced from switchgrass. This is achieved by pretreatment which is one of the most important steps in the utilization of lignocellulosic biomass for biofuel production. Current cost for pretreatment (up to 30 cents per gallon of ethanol) contributes a huge part of the overall cost of biofuel production from lignocellulosic biomass (Mosier *et al.*, 2005). Hence lowering the cost of pretreatment or even omission of this step has been an important research goal in the past couple of decades. Ammonia pretreatment is one of the many ways of pretreating switchgrass. Many other chemical pretreatments like ammonia fiber explosion method (AFEX) have the problem of chemical recovery and neutralization, and the release of inhibitory compounds. These can be overcome to a certain extent by washing and adjustment of pH (Kurakake *et al.*, 1999).

Autoclaving is typically done as a part of aseptic techniques to maintain a sterile environment before any experiment is performed. When switchgrass is being autoclaved, it is subjected to a high temperature (121°C). A hypothesis was suggested that autoclaving could release fermentable carbohydrates that *T. neapolitana* utilizes for fermentation. This hypothesis was tested by analyzing carbohydrates by HPLC after autoclaving. The results (Fig.10) indicated that autoclaving does release fermentable carbohydrates from switchgrass. Specifically, cellobiose is seen predominantly in the autoclaved samples. At a switchgrass concentration of 5 g/L, the glucose released by autoclaving was at a concentration of about 0.45 g/L. This is the substrate concentration which is usually used for switchgrass in all the experiments (5 g/L). This means that some glucose is liberated by autoclaving switchgrass and the concentration of these carbohydrates released is about 9% of the carbohydrate substrate concentration. In the case of non autoclaved samples, the concentrations of

carbohydrates released by just washing with distilled water are given on Fig 11. Xylose is predominantly seen in the non autoclaved samples. The concentration of glucose liberated by washing non pretreated 5 g/L switchgrass is 0.05 g/L as compared to 0.45g/L in the autoclaved samples. When the switchgrass concentration was increased to 50 g/L, the carbohydrate concentration increased to about 0.25 g/L which was still just 0.5% of switchgrass concentration.

These results show that while both autoclaving and just plain washing with distilled water release carbohydrates, the concentration of carbohydrates released is higher when autoclaved. Also, the type of carbohydrates released is different for the two processes – for instance, cellobiose is predominantly seen in autoclaved samples while xylose is seen predominantly in non autoclaved samples. Glucose and arabinose are seen in both autoclaved and non autoclaved samples.

Similar studies were performed on ammonium hydroxide pretreated switchgrass and the carbohydrates liberated were analyzed by HPLC. When ammonium hydroxide pretreated switchgrass was autoclaved, arabinose was the only sugar that was detected. When pretreated non autoclaved switchgrass was analyzed, no carbohydrates were detected. This shows that most soluble carbohydrates are washed off when switchgrass is pretreated with ammonium hydroxide and the subsequent washing. Many unidentified peaks were also obtained in this study.

***T. neapolitana* – Growth on Filter paper**

Cellulose is a renewable carbon source consisting solely of glucose units, and it is degraded by extracellular enzymes produced by various microbes. Fungi, including *Trichoderma*, *Penicillium*, and *Fusarium spp.* are efficient producers of cellulolytic enzymes. Cellulose can be chemically modified to be used in various applications (Itavaara *et al.*, 1999). These modifications sometimes affect its

biodegradability. Filter paper is conventionally used as cellulosic material for analysis of microbial activity.

When *T. neapolitana* was grown on filter paper, a yellow colored substance was produced, as shown in Fig.12, as the filter paper was being consumed. A yellow coloration has been previously observed with *Clostridium thermocellum* (Ljungdahl *et al.*, 1983) when it was grown on filter paper and cellobiose. This was termed as yellow affinity substance and was believed to be involved in the cellulose degradation. The yellow affinity substance has been postulated to aid the effective binding of endoglucanase to the cellulose fibers. It is interesting that such coloration was observed when *T. neapolitana* was grown on cellobiose too. The exact chemical structure of the yellow affinity substance is unknown, but it is believed to be a carotenoid-like compound of MW 1050– 1300 (Kopec̣ny' *et al.*, 1997). It has also been shown to be oxygen sensitive (Ljungdahl *et al.*, 1983; Kopec̣ny' *et al.*, 1997).

T. neapolitana was grown on filter paper to analyze its fermentation products- hydrogen (Fig. 13) and acetate (Fig. 14) over a period of 5 days. This experiment was also conducted to assess if *T. neapolitana*'s growth is affected by yeast extract. *T. neapolitana* is known to utilize various nitrogen sources. Nguyen *et al.* (2008) reported that hydrogen production for *T. neapolitana* increased as yeast extract concentration in the medium increased from 0.5 g/L to 4.0 g/L. Certain micronutrients, other than amino acids, present in yeast extract are used by the bacteria for better hydrogen production (van Niel *et al.*, 2002). Hence it was hypothesized that part of the hydrogen that was being produced by *T. neapolitana* is from yeast extract. Fig.13. shows the cumulative hydrogen production after five days of growth on filter paper.

About 4.5% hydrogen was made from just the medium, devoid of filter paper. The hydrogen produced could come from the yeast extract and trypticase present in the medium. It has been shown that yeast extract is essential for the growth of *Thermotoga* species (Balk *et al.*, 2002). The culture without any yeast extract produced only 2.8% hydrogen in the headspace. This hydrogen could have been made from the trypticase or the filter paper. In this medium with yeast extract, increasing concentrations of filter paper showed a corresponding increase in hydrogen production, it can be interpreted that a portion of the filter paper is being utilized. The effect of filter paper concentration and yeast extract on acetate production is shown in Fig 14.

Acetate was analyzed over a period of five days and the acetate concentrations went to about as high as 5 mM when the filter paper concentration was increased to 15 g/L. This shows that *T. neapolitana* ferments filter paper although yeast extract seems to be an essential component in the process.

Effect of light on *T. neapolitana* s growth in carbohydrates

T. neapolitana was isolated from a deep sea hydrothermal vent. In both the Pacific and the Atlantic Oceans, there are places where hot magma under the surface of the sea floor causes cracks in the Earth's crust. Sea water seeps into these holes, only to be forced out as mineral rich, warmed geysers into the cold, oxygen rich, and deep ocean water. These unique springs are known as hydrothermal vents. The primary source of light at deep-sea vents is thermal radiation due to the high temperature of the hydrothermal fluid. This thermal light peaks in the infrared range with a tail that extends into the visible (White *et al.*, 2002). Hence it can be assumed that for the most part, the

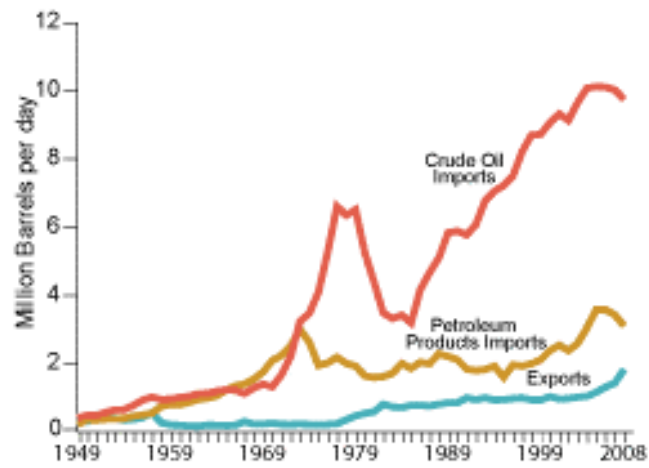
deep sea hydrothermal vent environment is in darkness. Since this is the natural habitat for *T. neapolitana*, it can be hypothesized that *T. neapolitana* might grow better when kept in dark. Growth rates were measured in *T. neapolitana* growing on different carbohydrates under light and absence of light. (Fig. 16)

The results suggest that *T. neapolitana* grew faster in the absence of light. The growth of *T. neapolitana* was best when grown in a mixture containing glucose, xylose and cellobiose followed by cellobiose and glucose. The growth was relatively slow when grown on glucose in the presence of light. Previously, Connors *et al.*, (2006), had shown that glucose is not the best substrate for *T. neapolitana*. This may be explained by differences in thermolability of these carbohydrates, apart from the metabolic variations. Further study on the differences in metabolism in the presence and absence of light is required to further interpret this behavior of *T. neapolitana*.

CONCLUSIONS AND FUTURE STUDIES

There could be many reasons for this difference in growth in the presence and absence of light by *T. neapolitana*. Infrared radiation could affect the metabolism, considering the fact that this organism was isolated from a hydrothermal vent. Further investigation into the effect of radiation on hydrogen production might help understand this phenomenon.

T. neapolitana has always been studied for its fermenting abilities. It has been shown to utilize complex carbon sources like peaches to produce 18% to 25% headspace hydrogen (Jain, 2009). But, its ability to hydrolyze complex lignocellulosic biomass is yet to be proven. With yeast extract contributing to most of the hydrogen being produced, and the degradation being slow and almost never complete, *T. neapolitana* cannot be employed in an economically viable method of hydrolyzing and fermenting switchgrass. Also, its ability to consume xylose may be studied in detail for *T. neapolitana* to still show promise in the hydrolysis area. The most interesting phenomenon that has been observed in this study is the preference of dark to light for a faster growth rate. This poses many questions about the physiology and ecological factors pertaining to *T. neapolitana* such as, what part of the radiation affects the metabolism? Is it the infrared radiation? Why is there the difference in conditions preferred? Could growing the bacteria in dark increase biomass? Further studies on the ecology and metabolomics of *T. neapolitana* could help the assessment of this bacterium for use in the biofuels industry.



Source: U.S. Energy Information Administration, *Annual Energy Review 2008*.

Fig.1. Trend in crude oil and petroleum imports from 1949 - 2008 in the United States.



Fig.2. Switchgrass (*Panicum virgatum*), (Enviroone crop, tree and plant database, <http://www.enviroone.com/CropDatabase.aspx>, (accessed April 23, 2010))

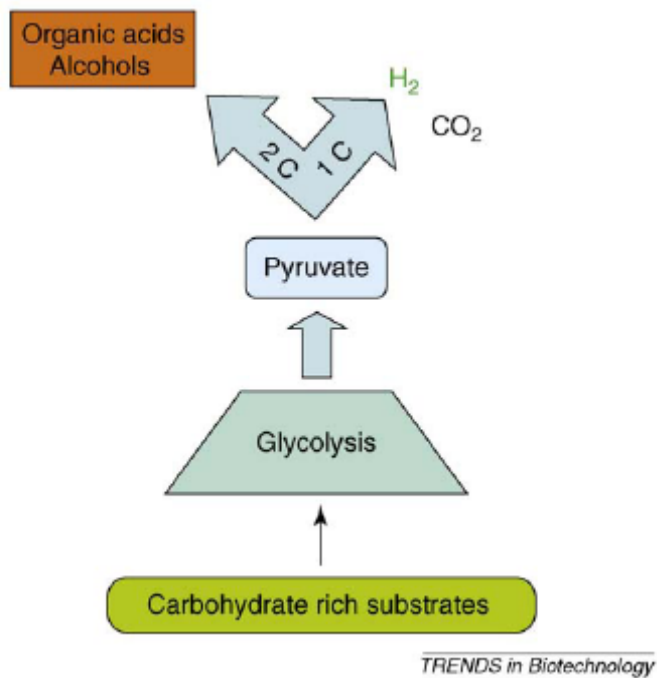


Fig.3. Fermentation of carbohydrate rich substrates to fermentation products like hydrogen, carbon dioxide and organic acids and alcohols (Hallenback *et al.*, 2009).

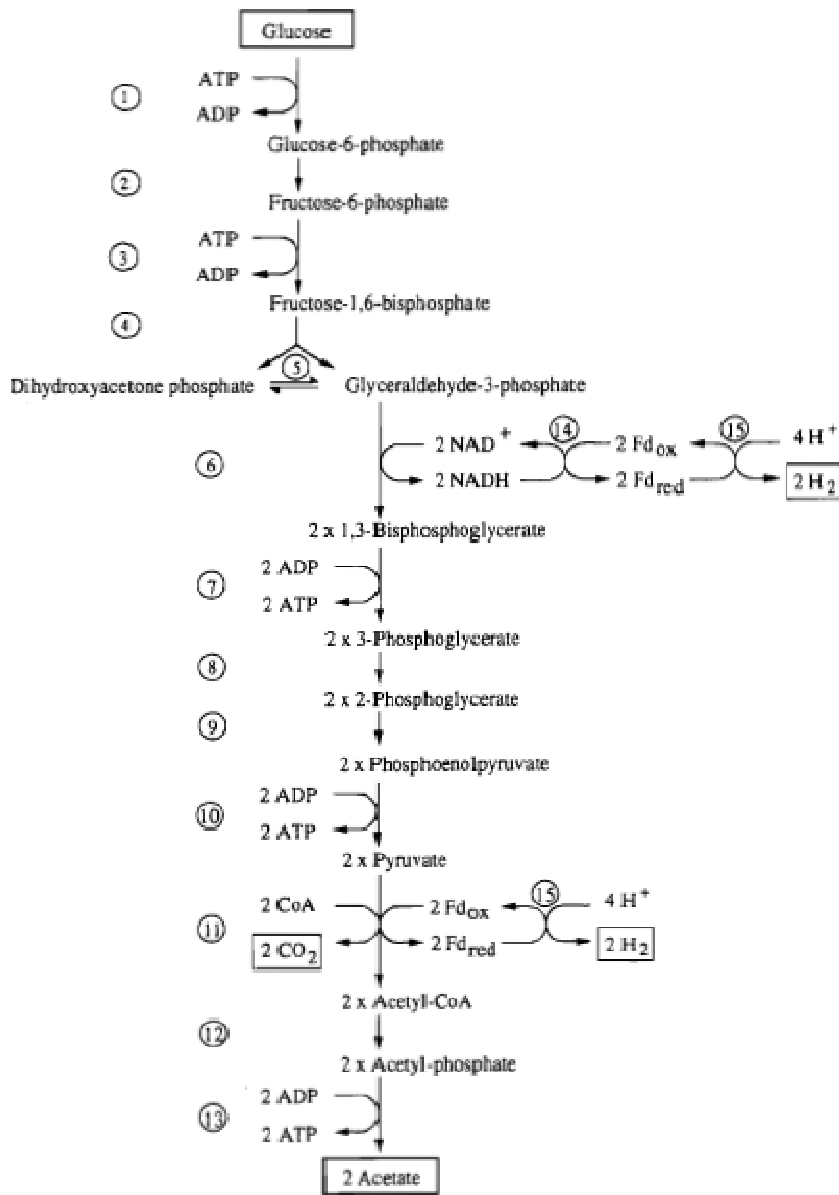


Fig.4. Metabolic pathway of glucose being fermented to 2 acetate, 2 CO₂ and 4 H₂ by *Thermotoga maritima* (Schroder *et al.*, 1994).

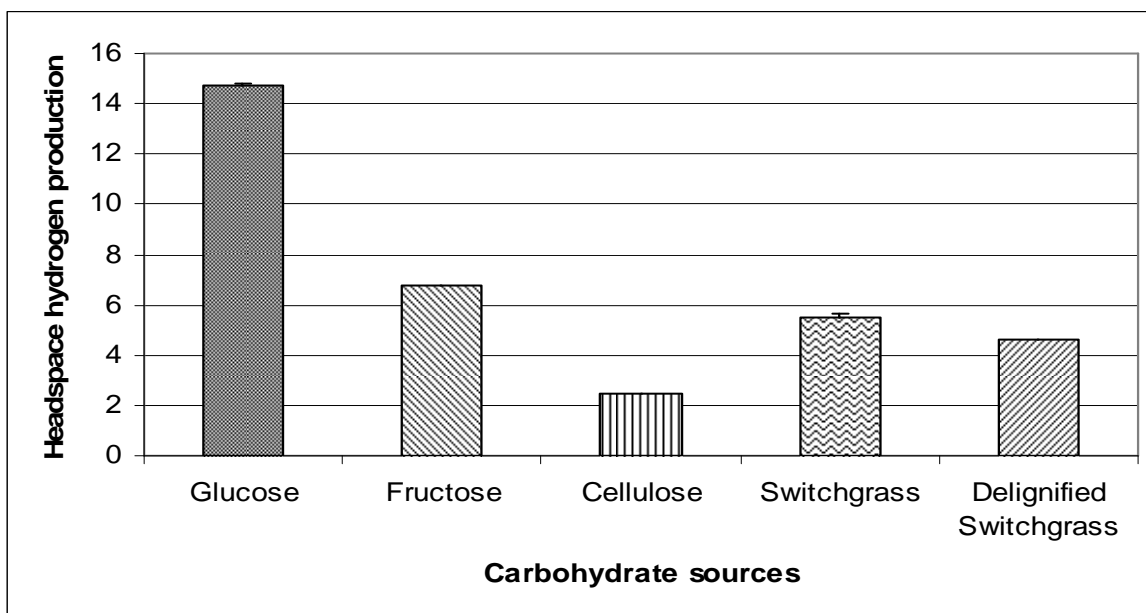


Fig.5. Headspace hydrogen production from various carbon sources.

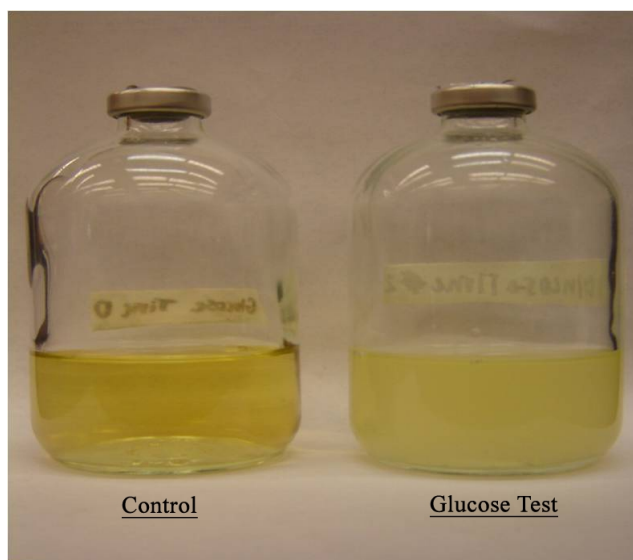


Fig.6. Glucose medium – uninoculated control and inoculated test sample.

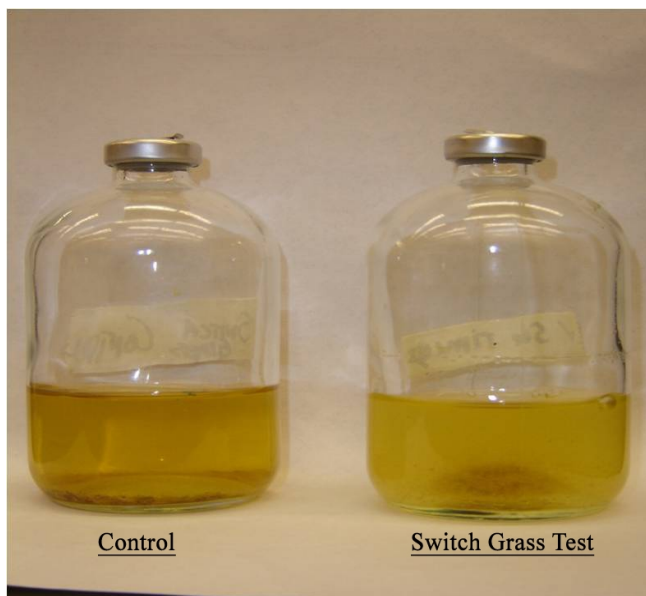


Fig.7. Switchgrass uninoculated control and inoculated test sample.

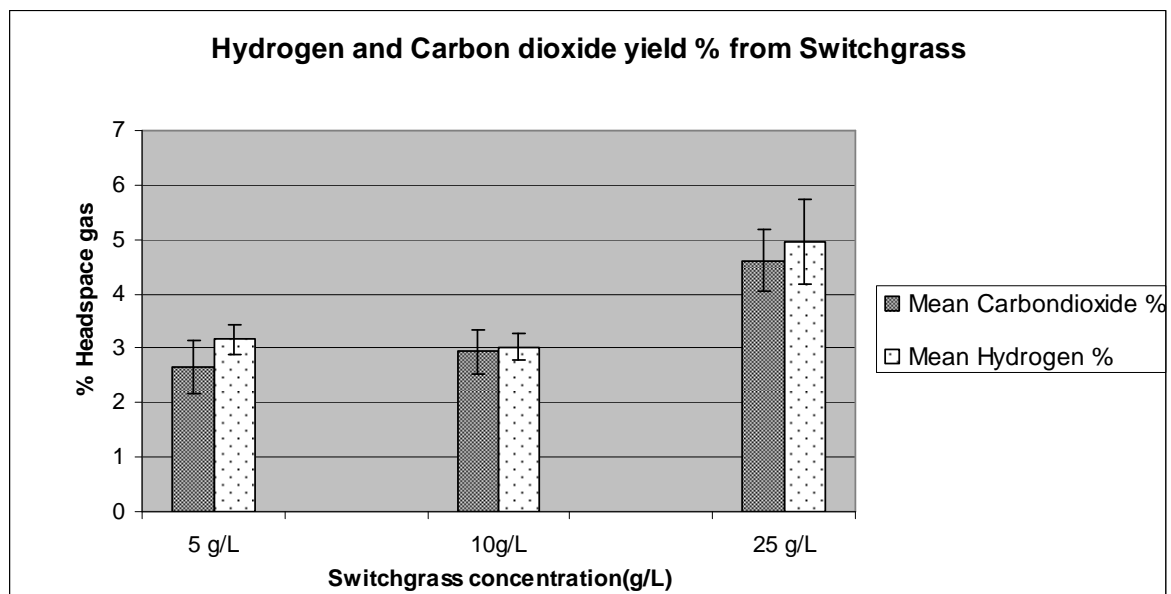


Fig.8. Hydrogen and carbon dioxide production from increasing concentrations of switchgrass.

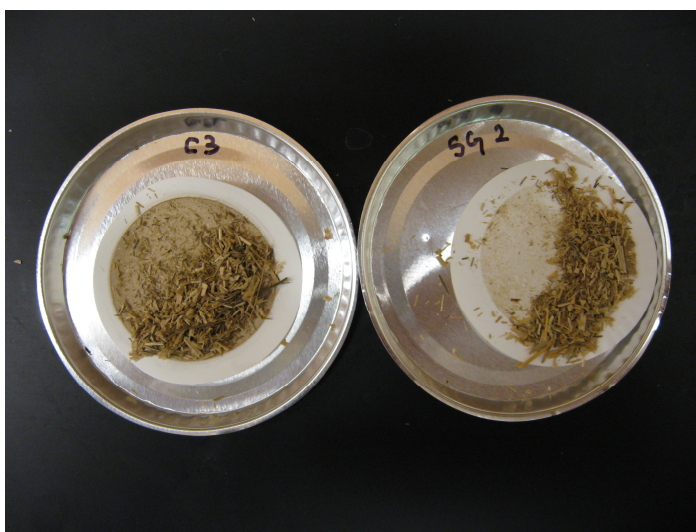


Fig.9. Filtered, dried switchgrass (uninoculated control on the left and inoculated on the right)

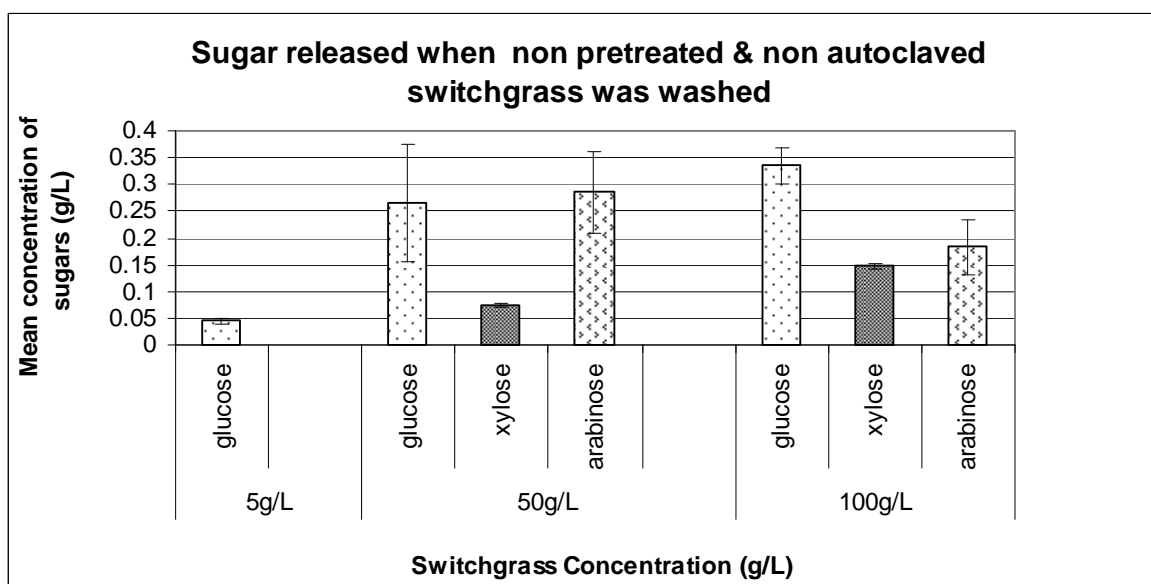


Fig.10. Carbohydrates released when non pretreated non autoclaved switchgrass was washed

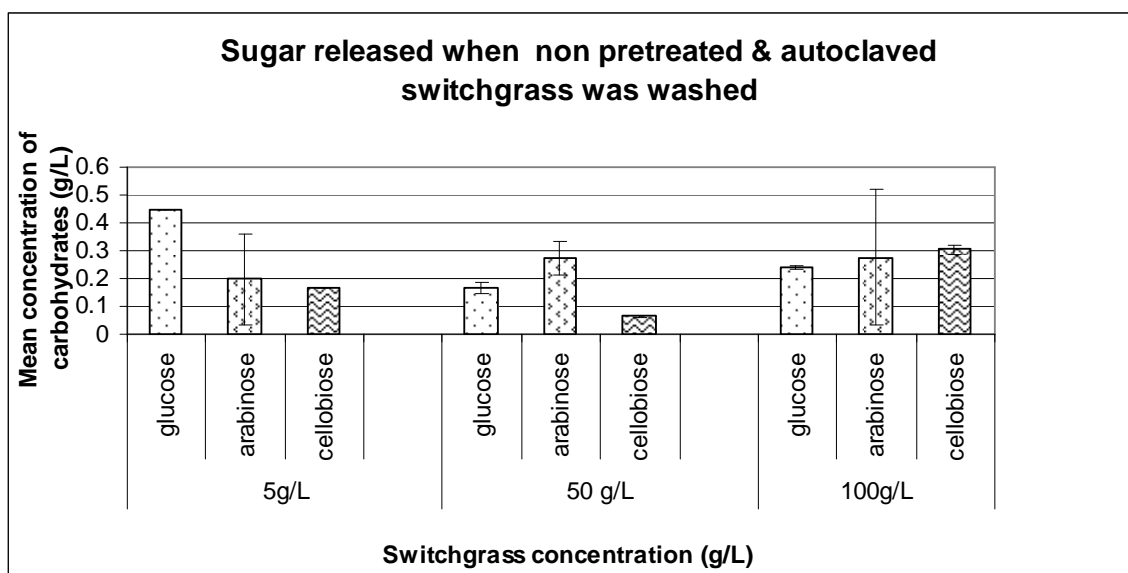


Fig.11. Carbohydrates released when non pretreated autoclaved switchgrass was washed.



Fig.12. Yellow color formed in *T. neapolitana* culture grown on filter paper, on the left. On the right is the uninoculated control.

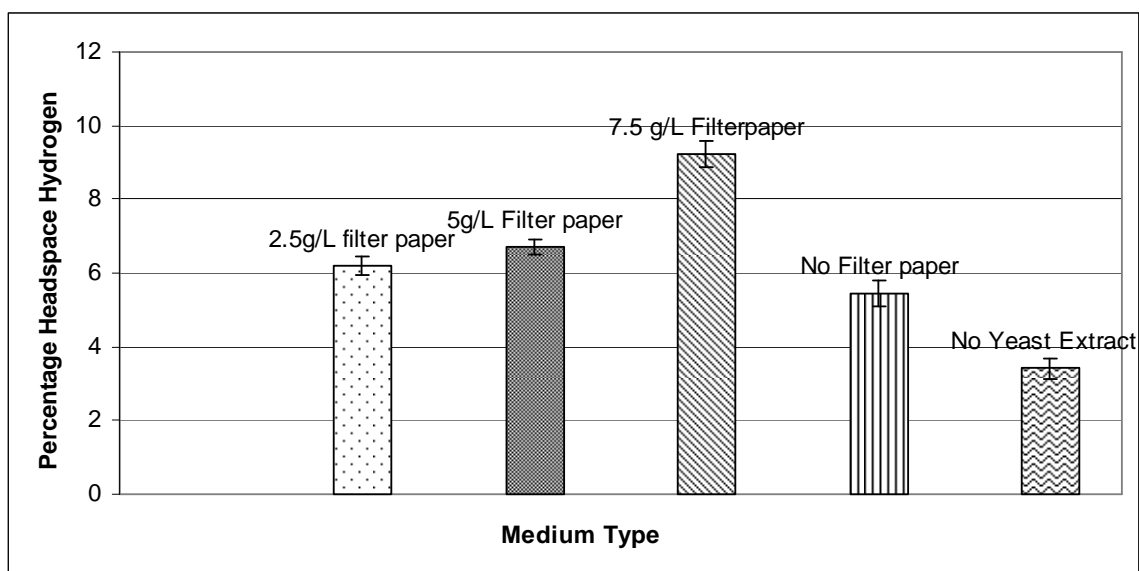


Fig.13. Percentage of headspace hydrogen produced from increasing concentrations of filter paper and medium with and without yeast extract and filter paper.

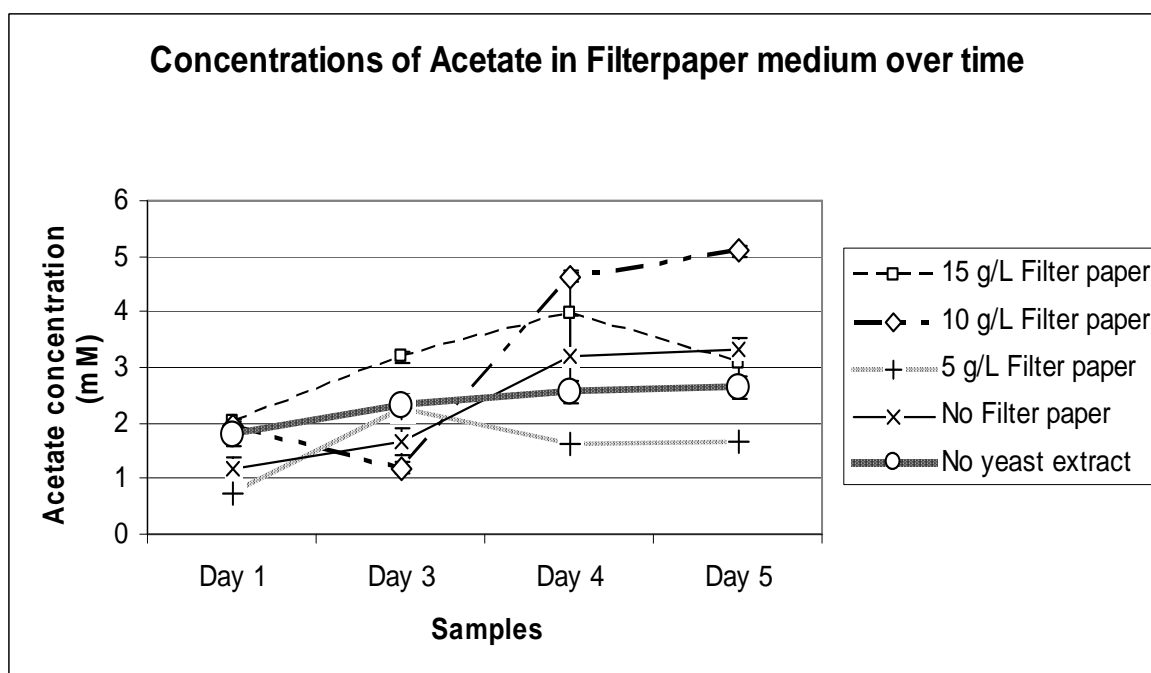


Fig.14. Acetate production from filter paper and yeast extract over a period of five days by *T. neapolitana*

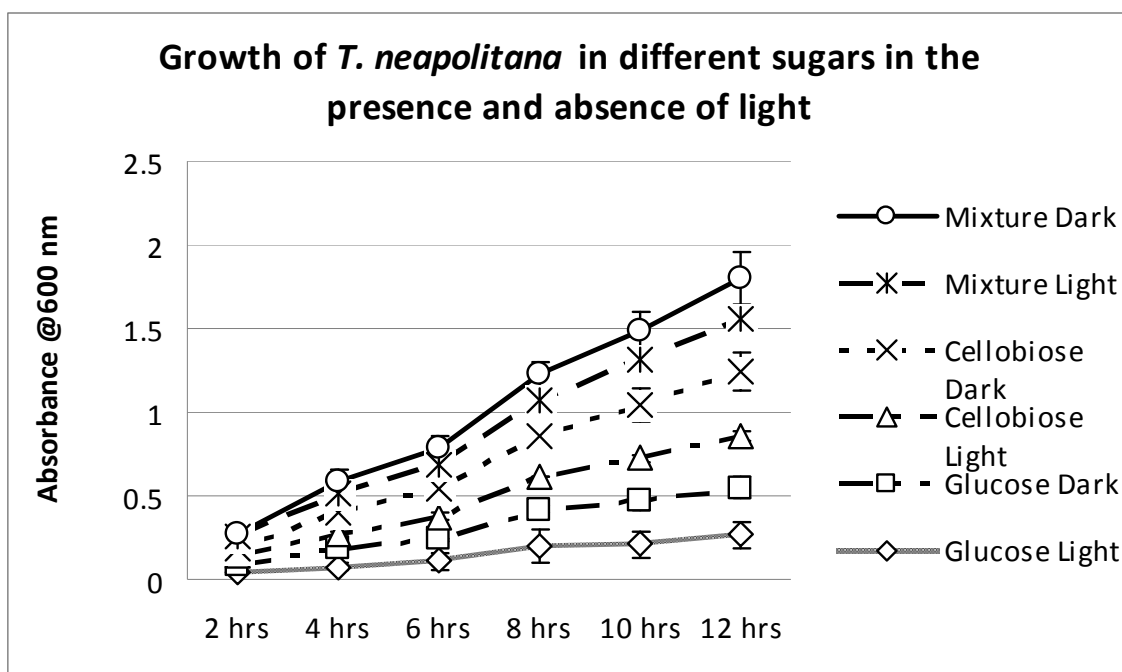


Fig.15. Growth rate of *T. neapolitana* when grown in the presence and absence of light

Biohydrogen system	Hydrogen synthesis rate(converted units)
Direct photolysis	0.07 mmol H ₂ /(l*h)
Indirect photolysis	0.355 mmol H ₂ /(l*h)
Photofermentation	0.16 mmol H ₂ /(l*h)
Dark fermentation- Thermophilic, undefined	8.2 mmol H ₂ /(l*h)
Dark fermentation- Extreme thermophilic, pure strain	8.4 mmol H ₂ /(l*h)

Table.1. Modified table from Levin *et al.*, 2003 showing different methods of hydrogen production and their corresponding hydrogen yields.

Mean Dry weight of switchgrass (control)	Mean Dry weight of switchgrass (inoculated)
0.241 g	0.218 g

Table.2. Table showing dry weight difference between inoculated and uninoculated switchgrass (mean of 3 values, T-test was performed (p value <0.05))

REFERENCES

1. U.S. Department of Energy Hydrogen program. Hydrogen production <http://www.hydrogen.energy.gov/production.html> (accessed April 23, 2010).
2. Enviroone crop, tree and plant database, <http://www.enviroone.com/CropDatabase.aspx>, (accessed April 23, 2010).
3. Energy Information Administration, U.S.DOE. June 2009. *Annual Energy Review. 2008*. DOE\EIA- 0384(2008) p.166.
4. Balk.M, W .Jan. and A. J. M. Stams. 2002. *Thermotoga lettingae* sp. nov., a novel thermophilic, methanol-degrading bacterium isolated from a thermophilic anaerobic reactor, *Int.J. Syst. Evol. Microbiol.* 52:1361–1368
5. Bisaria, V.S. 1998. Bioprocessing of agro-residues to value added products. In: Martin, A.M. (Ed.), *Bioconversion of Waste Materials to Industrial Products*, second ed. Chapman and Hall, UK.
6. Bok J.D, D.A., Yernool and Eveleigh D. E.1998. Purification, characterization, and molecular analysis of thermostable cellulases CelA and CelB from *Thermotoga neapolitana*, *Appl. and Environ.Microbiol.*, 54:4774–4781
7. Bronnenmeier, K., A.Kern.,W.Liebl, & W. L.Staudenbauer.1995.Purification of *Thermotoga maritima* enzymes for the degradation of cellulosic materials. *Appl. Environ. Microbiol.* 61:1399–1407.
8. Carere C. R., R. Sparling , N. Cicek and D.B. Levin. 2008. Third Generation Biofuels via Direct Cellulose Fermentation, *Int. J. Mol. Sci.*, 9: 1342-1360;
9. Chum, H.L., D.K., Johnson, S.Black, J.Baker, K. Grohmann, K.V Sarkanen, K. Wallace, H.A Schroeder,.1988. Organosolvent pretreatment for enzymatic-hydrolysis of poplars: 1. Enzyme hydrolysis of cellulosic residues. *Biotechnol. Bioeng.* 31 (7): 643–649.
10. Das D, T Nejat Veziroglu. 2001.Hydrogen production by biological processes: a survey of literature. *Int J Hydrogen Energy*; 26: 13–28.
11. Decker, S. R., W. S Adney, E.Jennings, T.B. Vinzant, M.E Himmel.2003. Automated filter paper assay for determination of cellulase activity. *Appl Biochem Biotechnol.* 105-108:689–703.

12. Duffard, G. Y., C. M. McCutchen, P. Leduc, K. N. Parker, and R. M. Kelly. 1997. Purification and characterization of extremely thermostable β -mannanase, β -mannosidase, and α -galactosidase from *Thermotoga neapolitana* 5068. *Appl. Environ. Microbiol.* 63:169–177
13. Elobeid, A., S. Tokgoz, C. Hart. 2007. The Ethanol outlook for Brazil and the United States and implications for livestock. *Int Sugar J.* 109: 174-177
14. Fardeau, M. L., B. Ollivier, B.K.C. Patel, M. Magot, P. Thomas, A. Rimbault, F. Roccchiccioli, J.L. Garcia.1997. *Thermotoga hypogea* sp. nov., a xylanolytic, thermophilic bacterium from an oilproducing well. *Int. J. Syst. Bacteriol.* 47: 1013-1019.
15. Gabelsberger, J., W. Liebl, and K. H. Schleifer. 1993. Cloning and characterization of β -galactoside and β -glycoside hydrolysing enzymes of *Thermotoga maritima*. *FEMS Microbiol. Lett.* 109:131–138.
16. Hallenbeck, P.C., D. Ghosh. 2009. Advances in fermentative biohydrogen production: the way forward? *Trends Biotechnol.* 27: 287–297
17. Hallenbeck P, JR. Benemann. 2002. Biological hydrogen production: fundamentals and limiting processes. *Int J Hydrogen Energy*; 27:1185–94
18. Hawkes FR, R. Dinsdale, DL Hawkes, I.Hussy. 2002. Sustainable fermentative biohydrogen: challenges for process optimization *Int J Hydrogen Energy*; 27:1339–47.
19. Huber, R., and K. O. Stetter.1992.The order Thermotogales, p. 3809–3815. *In* A. Balows, H. G. Tru"per, M. Dworkin, W. Harder, and K. H. Schleifer (ed.), *The prokaryotes*, 2nd ed. Springer-Verlag, New York, N.Y.
20. Huber, R., T. A Langworthy,, H.Konig, M. Thomm,, C. R.Woese, U. B Sleytr,, K. O Stetter.1986. *Thermotoga maritima* sp. nov. represents a new genus of unique extremely thermophilic eubacteria growing up to 90°C. *Arch. Microbiol.* 144: 324–333.
21. Jannesh, H.W., R. Huber, S. Belkin, K.O. Stetter 1988. *Thermotoga neapolitana* sp. nov. of the extremely thermophilic, eubacterial genus *Thermotoga*. *Arch. Microbiol.* 150:103-104.
22. Jeanthon, C., A.-L.Reysenbach, S. L'Haridon, A.Gambacorta, N.R.Pace, P. Glenat, and D.Prieur.1995. *Thermotoga subterranea* sp. nov., a new thermophilic bacterium isolated from a continental oil reservoir. *Arch. Microbiol.* 64: 91-97.
23. Kaplan, S., M.D. Moore 1998. Process for the production of hydrogen using photosynthetic proteobacteria. U.S. Patent 5,804,424.
24. Kelly, R.M., M.W.W. Adams, 1994. Metabolism in hyperthermophilic microorganisms. *Antonie van Leeuwenhoek* 66:247.

25. Keshwani. R, D. Jay, J. Cheng, 2009.Switchgrass for bioethanol and other value-added applications: A review, *Bioresource Technology* 100 1515–1523.
26. King, M. R., D. A. Yernool, D. E. Eveleigh, and B. M. Chassy. 1998. Thermostable α -galactosidase from *Thermotoga neapolitana*: cloning, sequencing and expression.*FEMS Microbiol. Lett.* 163:37–42.
27. Kopecný J. and B. Hodrova.1997.The effect of yellow affinity substance on cellulases of *Ruminococcus flavefaciens*, *Letters in Applied Microbiology*, 25:191–196
28. Kurakake M, W. Kisaka, K. Ouchi, and T. Komaki. 2001. Pretreatment with Ammonia Water for Enzymatic Hydrolysis of Corn Husk, Bagasse, and Switchgrass, *Appl. biochem.Biotech.*, 90.
29. Levin D.B., L.Pitt, M.Love.2004. Biohydrogen production: prospects and limitations to practical application, *International Journal of Hydrogen Energy* 29:173 – 185.
30. Ljungdahl.D.L, B.Pettersson, K- E. Eriksson, L. Wiegel.1983. A Yellow Affinity Substance involved in the Cellulolytic System of *Clostridium thermocellum*, *Current Microbiology*, 90.
31. Melis, A., Zhang, L., M. Forestier., M.L. Ghirardi, and M. Seibert. 2000.Sustained photobiological hydrogen gas production upon reversible inactivation of oxygen evolution in the green alga *Chlamydomonas reinhardtii*. *Plant Physiol.* 122:127–35
32. McLaughlin, S.B., J. Bouton, D.Bransby., B.V. Conger., W.R. Ocumpaugh, D.J.Parrish, C. Taliaferro, K.P.Vogel, S.D .Wulschleger,. 1999.Developing switchgrass as a bioenergy crop. In: Janick, J. (Ed.), *Perspectives on New Crops and New Uses*. ASHS Press, Alexandria, VA, 282–299
33. Nanavati, D., K.M Noll, and A. Romano, 2002. Periplasmic maltose- and glucose-binding protein activities in cell-free extracts of *Thermotoga maritima*. *Microbiology* 148:3531–3537.
34. Nelson, K.E., R.A.Clayton, S.R Gill, M.L.Gwinn, R.J Dodson, D.H Haft. 1999. Evidence for lateral gene transfer between Archaea and bacteria from genome sequence of *Thermotoga maritima*. *Nature* 399: 323–329.
35. Nowak, J., M Florek, W.Kwiatek, , J. Lekki, P. Chevallier, E. Zieba 2005. Composite structure of wood cells n petrified wood. *Matter Sci Eng*, 25: 119-130.
36. Ravot, G., M .Magot, M. L.Fardeau, B. K. C Patel, G. Prensier, A. Egan, J.L. Garcia,. B. Ollivier, (1995). *Thermotoga elfii* sp. nov., a novel thermophilic bacterium from an African oil-producing well. *Int. J. Syst. Bacteriol.* 45: 308-314.
37. Singh.A, and K. Hayashi.1995, Microbial cellulases: protein architecture, molecular properties and biosynthesis. *Adv. Appl. Microbiol.* 40:1–44.

38. Sunna, A., M. Moracci, M. Rossi, and G. Antranikian. 1997. Glycosyl hydrolases from hyperthermophiles. *Extremophiles* 1:2–23.
39. Takahata, Y., Nishijima, T. Hoaki, T. Maruyama. 2001. *Thermotoga petrophila* sp. nov. and *Thermotoga naphthophila* sp. nov., two hyperthermophilic bacteria from the Kubiki oil reservoir in Niigata, Japan. *Int. J. Syst. Evol. Microbiol.* 51:1901–1909.
40. Tarkow, H., W.C Feist. 1969. A mechanism for improving the digestibility of lignocellulosic materials with dilute alkali and liquid ammonia. *Advan.Chem. Ser.* 95:197-218.
41. Thring, R. W., E. Chorent, R. Overend.1990. Recovery of a solvolytic lignin: Effects of spent liquor/acid volume ratio, acid concentration and temperature. *Biomass.* 23(4): 289–305.
42. Turhollow, A.F., J.H Cushman, J.W Johnston.1990. Herbaceous Energy Crops Program: *Annual Progress Report for FY 1988*. ORNL - 6639 Oak Ridge National Laboratory
43. van Niel, E.W.J., M.A.W Budde, G.G de Haas, F.J van der Wal, P.A.M Claasen, A.J.M., Stams, 2002. Distinctive properties of high hydrogen producing extreme thermophiles, *Caldicellulosiruptor saccharolyticus* and *Thermotoga elfii*. *International Journal of Hydrogen Energy*, 27:1391-1398.
44. van Ooteghem Suellen A., 2002. Hydrogen Production by the Thermophilic Bacterium, *Thermotoga neapolitana*, *Appl. Biochem and Biotech.*98-100:1-9.
45. Vidal, P. F., J. Molinier.1988.Ozonolysis of lignin – improvement of invitro digestibility of poplar sawdust. *Biomass.*16 (1): 1–17
46. White, S. N., A. D. Chave, and G. T. Reynolds.2002.Investigations of ambient light emission at deep-sea hydrothermal vents, *J. Geophys. Res.*,107(B1).
47. Winterhalter, C., and W. Liebl 1995.Two extremely thermostable xylanases of the hyperthermophilic bacterium *Thermotoga maritima* MSB8. *Appl. Environ. Microbiol.* 61:1810–1815.
48. Wood, T. M., J. N.Saddler. 1988. Increasing the availability of cellulose in biomass materials.*Methods Enzymol.* 160:3–11.
49. Wright, L. 2007. Historical Perspective on How and why switchgrass was selected as a “model” high-potential energy crop. ORNL/TM-2007/109: Oakridge, TN: Oakridge National Laboratory
50. Wu, Z. W., Lee, Y. Y. 1997. Ammonia recycled percolation as a complementary pretreatment to the dilute-acid process. *Appl. Biochem. Biotechnol.* 63 (5): 21–34.
51. Wyman, C.E. 1994. Ethanol from lignocellulosic biomass: Technology, economics, and opportunities. *Bioresource Technology*, 50 (1): 3-15.

52. Yu, Xiaohui, 2007. Biohydrogen production by the hyperthermophilic bacterium *Thermotoga neapolitana*, Doctorate thesis, Clemson University, <http://gradworks.umi.com/32/90/3290693.html>, (accessed on April 23, 2010)